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Generation of small mutation in large genomic fragments by homologous recombination: description of the technique and examples of its use

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ABSTRACT

We have developed a technique of homologous recombination in bacteria which allows the mutagenesis of large genomic fragments cloned in cosmids. The desired mutation is first introduced into a plasmid clone and is then transferred to the appropriate cosmid clone by the means of double antibiotic selection coupled with phenotypic selection. We describe three different types of construct made by this technique.

INTRODUCTION

In studies of gene expression, it is important to be able to mutate small regions of cloned DNA in a directed and precise manner. This can be accomplished by removing or inserting small segments of DNA from plasmids by restriction enzyme digestion and ligation, or by site directed mutagenesis. These techniques are accomplished most easily in the context of small fragments of DNA cloned in plasmids; indeed, this is an absolute requirement for site directed mutagenesis (1). However, it is frequently necessary to reintroduce these mutated sections into, for example, genes cloned in cosmids and this is technically both difficult and imprecise, since it relies on the presence of naturally occurring restriction sites. We have developed a technique for modifying large genomic clones by homologous recombination in *E. Coli*. This technique is of widely applicable benefit since any small mutation can be introduced into any large genomic context without the need for particular restriction sites. We have used this technique to generate mutations in two human genes linked (in vitro) for use in transgenic mouse studies. It is often useful to be able to have two genes linked in tandem for expression studies, particularly where the genes are functionally related, for example, coding for different subunits of the same protein, or coding for proteins acting in the same pathway or expressed in the same tissue. In this case, the genes chosen were those for human α 1-antitrypsin (A1AT) and human retinol-binding protein (RBP) both of which are expressed in liver (2). RBP was present as an internal control for variation in A1AT expression.

We describe here three uses of the homologous recombination protocol: -the insertion of a short heterologous reporter gene, the mutation of short cis-acting elements in the A1AT promoter and the substitution of a single base pair in the A1AT coding region.

MATERIALS AND METHODS

Homologous recombination procedure

This protocol is a modification of that described by Poustka et al (3). The bacterial strain used for each step was *Escherichia coli* k12 rec A⁻ DH1 (4). Antibiotic selection was performed at 15 microgram/milliliter for kanamycin and 50 microgram/milliliter for ampicillin. In vitro packaging of cosmid DNA was done with Gigapack (Stratagene) extract according to the protocol recommended by the manufacture, with an average efficiency of 1×10^4 per microgram of DNA. The analysis of recombination in and out events was done by restriction mapping and Southern hybridization; in the cases of PM1 and EM3, the entire region of homology between donor and acceptor, after recombination, has been controlled by subcloning and sequencing by dideoxy-method (5). In the case of the PiZ construct, cosmids were analysed by probing Southern blots with 'M' and 'Z' specific oligonucleotides as described (6).

Several cases of inappropriate rearrangement within the cosmid insert, both in recombination in and out events, were observed and discarded.

Construction of plasmids and cosmids

pCos RBP/A1AT cosmid was obtained by ligation of the 18 kb SalI fragment from the RBP gene (7) and the 17.5 kb SalI fragment from the α 1-antitrypsin gene (A1AT73) (8), into the SalI site of pCos2 (3). This cosmid was used as acceptor in an homologous recombination reaction in *E. Coli* in order to insert the CAT coding region downstream of the RBP promoter. The donor was a pEMBL-CAT derivative containing the HindIII-ClaI segment from PSV2-CAT (9) flanked at the 5' end by a segment of the RBP gene, from N - 1650 to N + 36 and at the 3' end

by another segment of the RBP gene, from N +59 to N +448. The resulting cosmid (pCos RBP-CAT/A1AT) was used as acceptor in three independent reactions in which the donors were in the first two cases described pEMBL 18 containing the promoter of the A1AT gene from -261 to -37, carrying the EM3 or the PM1 mutations (10). In the third reaction the donor was pUC9 containing a 402 bp amplified fragment. This was derived by PCR of DNA from an individual homozygous for the PiZ mutation. The primers were as described (6). The product was cloned into the SmaI site of the vector by blunt-ended ligation. The insert was sequenced using a double-stranded sequencing protocol and was found to be identical to that of the published sequence of A1AT (11), apart from the G-A substitution characteristic of the PiZ allele.

RESULTS

General principle of the technique

In order to insert or delete sequences within the context of large DNA segment we set up an *in vivo* homologous recombination protocol, partially based on the work of Cesareni et al (12) and Poustka et al. (3) which is outlined schematically in fig 1 and described in more detail in materials and methods. The gene to be modified is cloned in pCos2, a cosmid vector previously described (3), carrying the kanamycin resistance gene. The salient feature of pCos2, which is essential for this technique, is the lack of any sequence homology with plasmids of the pUC (13) or pEMBL families (14). A short (200–2500 nucleotides) DNA sequence of the gene containing the desired mutation is cloned in the plasmid which has the ampicillin resistance gene. The salient feature of these vectors, is that 1) they do not contain any sequence homologous to pCos2, so that the only homology is within the test gene; 2) they carry a lac operator sequence. The lac operator serves the purpose of conferring a phenotype to any bacterial colony harboring a recombinant. This is because the lac operator binds avidly to the lac repressor, causing the derepression of the bacterial lac operon, with consequent high amounts of beta-galactosidase activity, which can be easily detected on X-gal plates. In the homologous recombination experiment the recombinant plasmid functions as donor and the pCos2 derivative cosmid functions as acceptor. It is thus possible to infect bacterial cells harboring the donor plasmid, with packaged acceptor cosmid (fig. 1, step 1). After a few hours of growth cells are harvested and the DNA extracted and packaged. After a second round of infection (fig. 1, step 2), cells are plated on kan-amp-Xgal plates, to select and identify the product of the single homologous recombination events (which yield blue colonies) carrying the donor plasmid 'recombined in' into the pCos2 acceptor; usually between 1×10^{-2} and 10^{-4} of the colonies are blue, apparently depending on the size of the region of homology between donor and acceptor constructs as described in detail below. The reverse reaction, a 'recombination out' of the plasmid sequences, occurs at a frequency between 10^{-2} to 10^{-3} . In practice blue colonies are grown for few hours, than DNA is extracted and packaged. In a third round of infection, cells are plated on kan-X-gal plates: colonies harboring the pCos2 DNA, without any plasmid sequences, are white because the bacterial lac operon is normally repressed. As shown in fig. 1., step 3, there are two possible products of this 'recombination out' event: A) the restoration of the original wild-type pCos2 construct, which is the unwanted product and B) the formation of a new construct carrying the

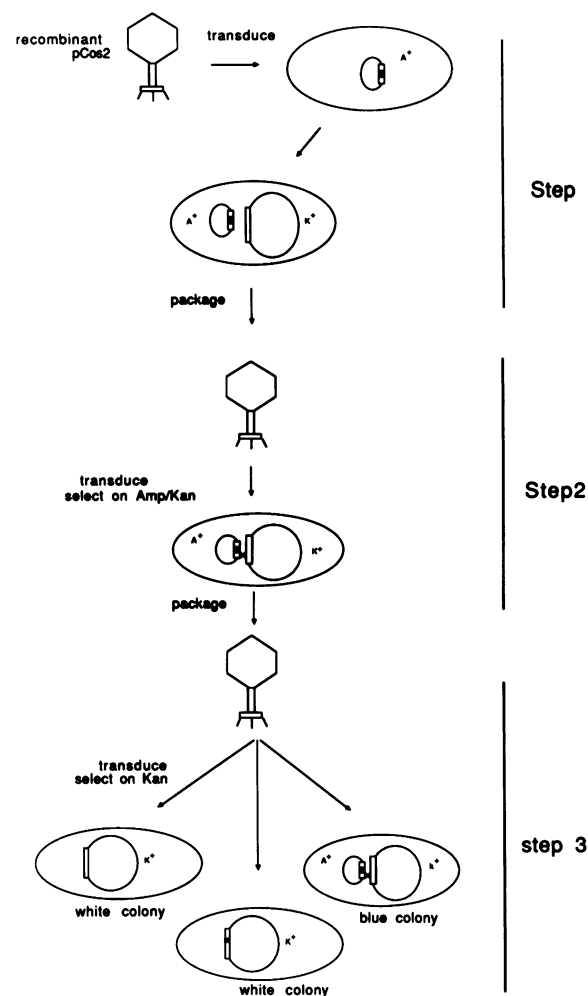


Fig 1. Homologous recombination protocol. *Step1.* The packaged recombinant cosmid is transduced into a bacterial cell harbouring the 'donor' plasmid carrying the desired mutation. The regions of homology between donor and acceptor varied from experiment to experiment (from 200 to 2000 nucleotides). Infected cells are grown for 4 hours in the presence of kan and amp, then are harvested and DNA is extracted and purified. *step2.* DNA, containing donor plasmid, acceptor cosmid and the cointegrate of donor and acceptor, is packaged *in vitro* and transduced into bacterial cells. The free donor plasmid cannot be packaged, whereas the free acceptor cosmid is counterselected in the presence of ampicillin, therefore the only colonies carrying the cointegrate can grow in the presence of both kan and amp. *Step3.* DNA is extracted and analyzed by restriction mapping and Southern blot in order to eliminate incorrect or rearranged cointegrates. The expected and verified cointegrate DNA is packaged again and transduced into bacterial cells in the presence of kanamycin and X-gal. Colonies carrying the product of the 'recombination out' event are white and are analysed for the presence or absence of the desired mutation.

desired mutation, which is the wanted product. A and B can be easily distinguished by colony hybridization with specific labelled oligonucleotide probes (see fig. 3).

Details of constructs made

The CAT coding region was inserted into the human RBP gene. The donor plasmid was a pEMBL-CAT derivative containing the CAT coding sequence bracketed by about 1700 bp 5' and about 400 bp 3' of the human RBP gene. The extent of homology between the inserts in this case was about 2100 bp, and the frequency of 'recombination in' events, i.e. double antibiotic resistant colonies, was 1/100. Of these, twelve were selected at

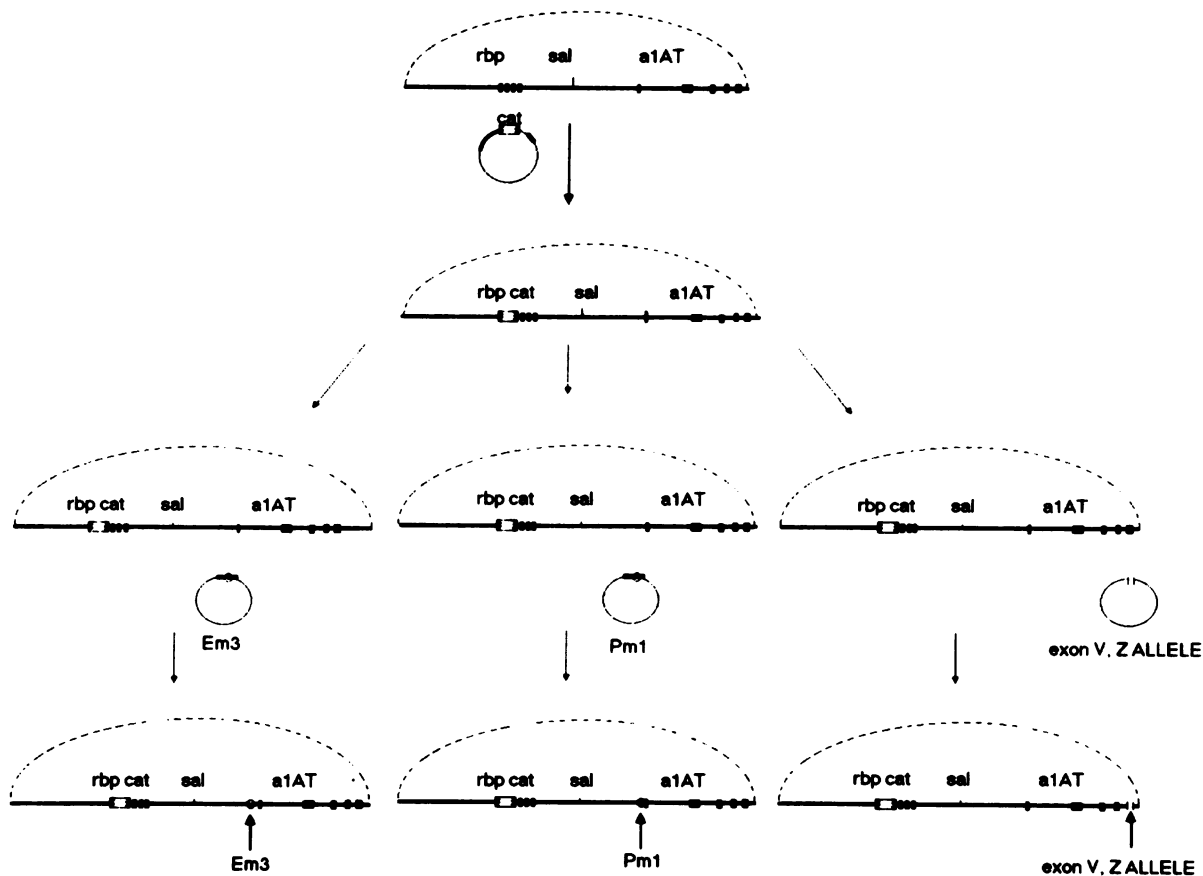


Fig 2. Constructs made

random, amplified and analysed by restriction mapping with EcoR1 and BamH1 independently. These enzymes give in the order of ten bands each. Out of these twelve clones, eight gave restriction patterns characteristic of the predicted integration into the first exon of the RBP gene. The other four clearly had large abnormal deletions, resulting in a restriction pattern which defied analysis. One of the clones which had undergone the predicted integration event was selected and grown in the presence of kanamycin alone. DNA was extracted, packaged again and plated on Xgal/Kanamycin plates. Of the resulting colonies 1/100 was white. Ten of these were selected by colony hybridization with the CAT specific probe and analyzed further by restriction mapping and blotting. All of them showed the predicted pattern for a correct replacement of the first exon of the RBP gene with the equivalent exon containing the CAT gene.

The resulting cosmid recombinant, pCos-RBP-CAT/A1AT, was used as acceptor for the recombination with DNA segments carrying mutations in the A1AT promoter. For the construction of these mutants, the plasmid donors were pEMBL derivatives containing a 224 bp fragment of the A1AT promoter. The mutations involved were PM1 and EM3 (10). In the case of PM1 the extent of homology was 220 bp and the frequency of 'recombination in' events was 1/3000. Ten of these colonies were checked and nine were found to have the predicted restriction pattern. One of these was grown and 60,000 bacterial colonies were tested on Xgal plates. Six consistently white clones were isolated, all of which had undergone the correct excision event, and two of which were recombinants as judged by colony hybridization and Southern blotting of restriction digests. The identity of the recombinants was verified further by the cloning

and sequencing of the entire region of homology (see materials and methods). In the case of EM3 the extent of homology was 218 bp and the frequency of 'recombination in' event was 1/10,000. One colony was grown and 10,000 bacterial colonies were tested on Xgal plates. Ten white colonies were selected all of which had undergone a correct event of 'recombination out'. Analysis of these clones showed five of them to be the desired constructs.

For the construction of the PiZ clone, a 402 bp fragment was amplified by PCR using primers which span exon V plus a small amount of flanking sequence (6), from DNA of a homozygous PiZZ individual. PiZZ homozygotes have a deficiency of serum A1AT due to the build-up of the mutant protein in the hepatocytes (15). This fragment was cloned into pUC9 and used as a donor in a reaction in which pCos-RBP-CAT/A1AT was used as an acceptor. The frequency of recombination in events was 1/2000. Of twenty colonies checked one showed the predicted restriction pattern. This was selected for the 'recombination out' event which occurred at a frequency of 1/500. Of ten colonies checked all had undergone the correct excision event. These were checked by hybridization with both M and Z specific oligonucleotides (16) and one was found to be the desired Z clone. See fig 2 for the hierarchy of the clones described above and fig 3 for an example of the screening technique used for the 'recombination out' event.

Orientation of region of homology

The orientation of the region of homology which determines the orientation of origin of replication in the plasmid relative to the origin of replication in the cosmid does not appear to be of vital importance for the success of this technique. This was established

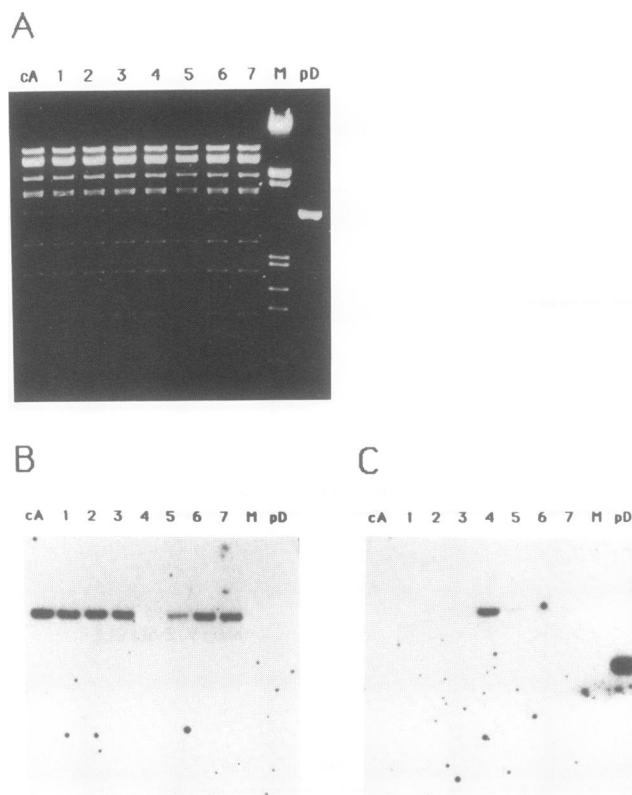


Fig3. Example of the screening technique used for the 'recombination out' event. cA: acceptor cosmid (RBP-CAT/A1AT); pD: donor plasmid (exon V of PiZ A1AT allele); M: Size Marker; 1-7 Cosmids which have undergone to the homologous recombination protocol. Panel A: EcoRI digests. Panel B: Southern blot analysis of the gel shown in panel A probed with M specific oligonucleotides. Panel C: same filter rehybridized with Z specific oligonucleotides. Clone 4 is the desired product.

by cloning the insert containing the PM1 mutation in both orientations in a plasmid. In each case, successful integration was obtained. However, in one case we observed that the colonies were unusually slow in growing after the integration event. These colonies were derived from the integration event in which the two origins of replication were in opposite orientations.

DISCUSSION

We have used the homologous recombination method to generate examples of several different types of construct which can be made using this technique. Obviously, there are other fields of research which could benefit from applications of this technique. Because we have not exploited this technique fully enough to provide a basis for statistical analysis we are not yet in a position to make firm conclusions as to which features of this protocol most influence the frequency of the desired recombination events. Nevertheless, the technique was successful in each case described, and furthermore the frequency of desired events (between 10^{-2} and 10^{-4}) is high enough to present no real problem given that we are dealing with an easily assayable event in bacterial cells. The amount of work involved is no greater than for many other cloning techniques. Whilst we are not able to establish precisely the ideal size requirements of the fragments to be used in the technique, achieved successful homologous recombination with areas of homology ranging from 218 bp to 2100 bp.

The technology for the mutagenesis of small fragments has been available for some time and has been widely exploited. However, the need to replace the mutagenized fragments in large genomic clones has often been a limiting factor. The system described in this paper for the relatively easy substitution of mutagenized fragments of DNA has potential application in several areas, as we have shown. Firstly it can be used to generate mutations, ranging from bp substitution to large deletions, in the regulatory regions of genes in cosmids to be used in studies of gene expression in cells or in transgenic mice. Here, the bacterial C-AT coding region was inserted into the first exon of the RBP gene using homologous recombination, an example of the use of this technique for inserting reporter genes into the context of a full length eukaryotic gene. We also generated a five bp substitution in the LF-A1 binding site, and a four bp substitution in the LF-B1 binding site of the A1AT promoter (17) by homologous recombination; this enabled us to use this technique to cause small alterations in cis-acting elements whilst leaving the rest of the gene intact. We were able to use the resulting cosmids in transgenic mice, to study the effect of the disruption of the binding sites for trans-acting factors without having any alteration in the rest of the gene and its flanking sequences.

Secondly the technique can be useful in the generation of small mutations, which have biological significance, in the coding region of genes. We replaced exon five of a PiM A1AT clone with the corresponding exon from a PiZ allele. Other genes may be mutagenized in this way, for example to interfere with signals within genes concerned with splicing or termination. In all, we believe that this technique will have applications in many varied fields.

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